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Enhanced metabolic flux of methylerythritol phosphate (MEP) pathway by overexpression of *Ginkgo biloba* 1-Hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate Reductase 1 (GbHDR1) gene in poplar

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Abstract

Terpenoids are of great interests in a broad range of health-beneficial biological activities and various industrial applications. In plants, terpenoids are synthesized by two distinct pathways, methylerythritol phosphate (MEP) and mevalonate pathways in a separate location. MEP pathway supplies isoprene precursors isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) of terpenoid biosynthesis in plant plastids. The MEP pathway has been an engineering target to increase the metabolic flux towards higher terpenoid production in plants. 1-Hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate reductase (HDR) is the terminal step of the MEP pathway to regulate the terpenoid biosynthesis and is encoded by three paralogous genes in *Ginkgo biloba*. In this study, we assessed the effect of overexpression of *GbHDR1* on terpenoid metabolism in poplar plants. Overexpression of *GbHDR1* in poplar plants accelerated growth and delayed winter-bud formation. Transcript levels of gibberellin, chlorophylls, and carotenoid biosynthetic genes in *GbHDR1*-overexpressing (*GbHDR1*ox) poplars were up-regulated, suggesting metabolic flux enhancement. Moreover, enhanced contents of chlorophylls and carotenoids in the leaves of the *GbHDR1*ox plants resulted in a higher photosynthetic rate as a consequence. Therefore, we expect the *GbHDR1* overexpression will be a desirable engineering point of the MEP pathway for enhancing terpenoid metabolic flux and production in plants.

Keywords: *Ginkgo biloba*, 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate reductase (HDR/IDS), Methylerythritol phosphate (MEP) pathway, Poplar, Terpene biosynthesis

Introduction

Terpenoids, also known as isoprenoids, are the largest group of natural products comprising more than 50,000 known structures. They play an essential role not only in plant physiology by participating in photosynthesis and hormonal processes but also in plant ecology by mediating various plant-herbivore, plant-pollinator, and plant-pathogen interactions [1]. Besides their natural roles, plant-derived terpenes have been found applications in pharmaceuticals, cosmetics, and as starting materials for other valued chemicals [2, 3]. In addition, they have recently shown promise as advanced

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biofuels because the properties of terpene-based biofuels have great potential to replace current fossil fuels [4]. Such needs for terpenoids from industry prompted researchers to seek means to use living organisms as cell factories. The diverse structures of terpenoids originate through the myriad rearrangement pathways of linear precursors originating from the condensation of surprisingly simple isoprene precursor units, isopentenyl diphosphate (IPP), and its isomer dimethylallyl diphosphate (DMAPP) [5]. To produce two isoprene precursors, plants use two separate pathways, the methylerythritol phosphate (MEP) pathway and the mevalonic acid (MVA) pathway. The former path resides in plastids [6], and the latter in peroxisomes and the cytosol [7, 8] in plants. In addition to the different localization, the role of each pathway is also distinctive. In general, the MEP pathway supplies building blocks for the mono- (C10), di- (C20), and tetraterpenes (C40), whereas MVA pathway supplies building blocks for sesqui- (C15) and triterpenes (C30).

Ginkgo biloba is one of the most ancient gymnosperms still surviving in the world. A special standardized, and purified extract (EGb 761) of *G. biloba* leaves that contains diterpenoids, ginkgolides and bilobalide as principal active components has been widely used as a pharmaceutical or health food with its efficacy affirmed by comprehensive pharmacological and clinical investigations [9]. MEP pathway genes from *G. biloba* have been cloned and characterized [10–15]. Among them, *GbDXS*, *GbCMK*, and *GbHDR* occur as small gene families with 2 to 3 paralogous members. The MEP pathway steps catalyzed by the family of isozymes are suggested as the important regulation points bifurcating the pathway into primary and secondary metabolisms [12, 14, 15].

The plastidial MEP pathway synthesizes isoprene precursors more economically than the MVA pathway by utilizing the carbon source directly from the Calvin cycle. The HDR is responsible for concluding the MEP pathway by converting 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate into IPP and DMAPP (Fig. 1). Being the terminal enzyme in the MEP pathway, HDR was suggested as a key regulator of terpenoid precursor production in the MEP pathway [16, 17]. Kim et al. [15] advocated functional differentiation among *HDR* paralogs of *G. biloba* by the promoter study in *Arabidopsis* by demonstrating the organ-specific expression of HDR homologs [18].

Frequently, the productivity of the target terpene product in plants is very low and economic accumulation needs longer period of cultivation. However, the presence of two separate terpenoid biosynthesis pathways in plants, could provide an advantage over the microbial system to siphon isoprene precursors from one of the pathways without adversely affecting overall terpene-related pathways. Apparently, only one terpene precursor pathway in microbial systems demand highly extensive

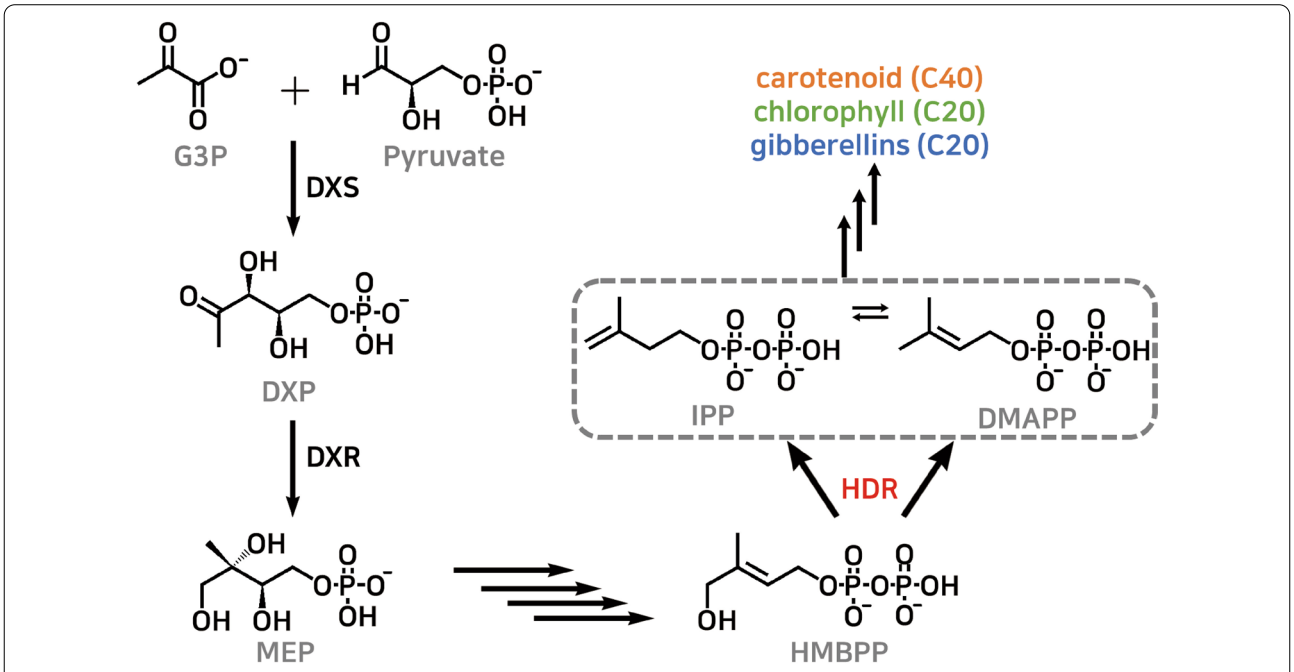

Fig. 1 Simplified MEP pathway in the plant cell. Involvement of the MEP pathway in the biosynthesis of diterpenoid plant hormone GA and photosynthetic pigments are emphasized

pathway manipulation to enable the microorganism withstand heavy metabolite diversion from normal isoprene precursor metabolism. Therefore, metabolic engineering of plants could provide more suitable production systems to utilize diverse terpene synthetic pathways to achieve high yield of the end-product terpenes. Terpene pathway-engineered plants, however, have seen few commercial successes to economically produce industry-relevant chemicals even though engineered microorganisms already have several lists of commercial successes [21].

In the present work, enhancement of terpenoid metabolic flux through the MEP pathway was successfully accomplished in poplar plants. Poplar is one of the most studied model plants and has been extensively used in plant metabolic engineering as promising bio-mass feedstock [19, 20]. To achieve this goal, we overexpressed one of the terminal enzymes, GbHDR1 from *G. biloba* in poplar, and the consequence of the approach was assessed in terms of downstream gene transcription. Focus was placed on genes involved in the biosynthesis of photosynthesis-related pigments and terpene-derived plant hormones. The up-regulation of genes in photosynthetic pigments and gibberellin (GA) biosynthesis positively affected the photosynthetic rate and biomass accumulation in the *GbHDR1*ox transgenic poplar plants.

Results

Enhanced growth performance in *GbHDR1*ox poplar plants

*GbHDR1*ox transgenic poplar seedlings, which were transferred from media plates into soil, showed rapid growth (Fig. 2A) compared with the wild type poplars (WT). At 8 weeks after potting, the height of *GbHDR1*ox poplars reached 24.6 cm, which is approximately 25% taller than of WT (19.6 cm) and had about 2 more leaves (16 versus 18.2 leaves for WT and *GbHDR1*ox) (Fig. 2B). All poplar plants were moved to the outdoor nursery after 8 weeks potting, and we regularly checked the phenotype of poplar plants. After 10 weeks of growing in the outdoor nursery, we found winter buds in the terminal shoots of all WT poplar plants (Additional file 1: Fig. S1A), but not in *GbHDR1*ox plants (Additional file 1: Fig. S1B). When the winter bud formed in all *GbHDR1*ox poplar plants, they were approximately 7% taller than WT (WT: 0.74 m *versus GbHDR1*ox: 0.79 m) (Additional file 1: Fig. S1C).

Increased terpenoid metabolic flux in *GbHDR1*ox poplar plants

We analyzed transcript levels of downstream genes relate to terpenoid metabolism in plastids such as isoprene, chlorophylls, carotenoids, and gibberellins (Fig. 3). Isoprene is one of the major terpene products in poplar leaves, and isoprene synthase (IS) is the only enzyme capable of isoprene synthesis that is believed to be emitted to cope with stress [22]. In *GbHDR1*ox transgenic poplars, a fivefold increase of *IS* transcript was observed (Fig. 3B). We also checked transcript levels of genes for photosynthetic pigments, chlorophylls and carotenoids. The transcript levels of chlorophyll synthesis-related enzymes, chlorophyll synthase (*CHS*) and chlorophyll a oxidase (*CAO*), as well as a key enzyme in carotenoid synthesis, phytoene synthase (*PSY*), were all up-regulated by 35%, 53% and 280% respectively. However, no statistical significances were found due to the high variations in transcript levels of *CHS*, *CAO* and *PSY* among the transgenic poplar plant lines (Fig. 3B). There were no transcript decreases of *CHS*, *CAO* and *PSY* in transgenic poplars plants. GAs are the diterpene-type plant hormones with a key intermediate of GA biosynthesis being ent-kaurene. In transgenic poplar plants, while the transcript levels of GA synthesizing and activating genes, ent-kaurene synthase (*KS*) and GA20-oxidase (*GA20ox*) increased by 170% and 85%, respectively, the level of GA2-oxidase (*GA2ox*), which inactivates GA, decreased by 40% (Fig. 3B).

Fig. 2 Typical phenotype of WT and the *GbHDR1*ox transgenic poplars. **A** WT and *GbHDR1*ox poplar plants 6-week after potting, **B** Height and leaf number of WT (n = 3) and *GbHDR1*ox poplars (8 lines, n = 3 to 5) plants. The asterisk symbol indicates **P < 0.01 and *P < 0.05

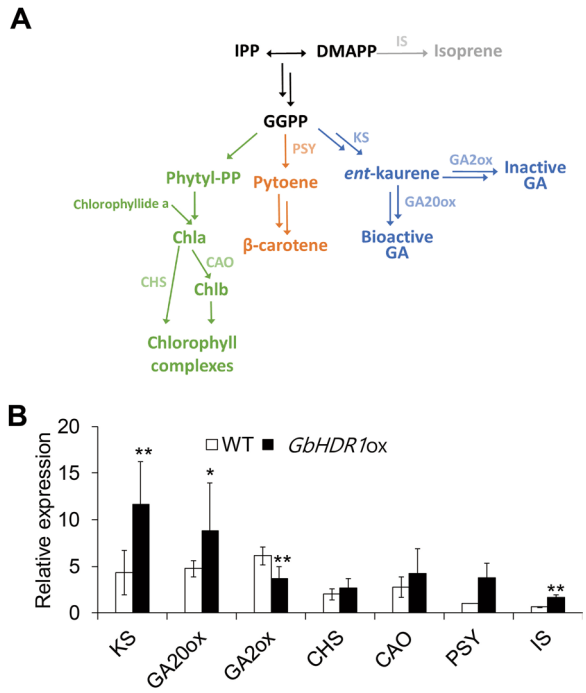

Fig. 3 Transcript levels of downstream terpene biosynthetic genes in poplar plants. **A** Overall scheme of terpene biosynthetic pathways and related enzymes, **B** comparison of transcript levels in WT (n = 3) and *GbHDR1*ox poplars (8 lines, n = 3 to 5). The white and black bar represents the WT and the transgenic plants, respectively. Error bar indicates standard deviation. The asterisk symbol indicates **P < 0.01 and *P < 0.05

Increased terpenoid photosynthetic pigments and photosynthetic rates

To verify the correlation of increased terpene-related transcript levels with the actual terpenoid contents in *GbHDR1*ox plants, we measured the contents of photosynthetic pigments, chlorophylls and carotenoids. Overexpression of *GbHDR1* in poplar clearly resulted in enhanced chlorophylls and carotenoids contents accompanied by elevated transcript levels of related genes mentioned in the previous section. We found the enhancement of chlorophyll a and b contents by 24% and 40% (Fig. 4A), and carotenoid contents were enhanced by 20% in *GbHDR1*ox poplar plants compared with WT (Fig. 4B).

As a result of increased photosynthetic pigment contents, the measured photosynthetic rates of the *GbHDR1*ox transgenic poplars under the given condition were higher by 30% compared to those of the WT plants (Fig. 4C).

Improvement of cell development in *GbHDR1*ox poplar plants

GA has been reported to have an effect on cell development in the secondary xylem in particular [23]. In order to confirm the hormonal effect in plant cell development, we performed phloroglucinol (PG) and berberine-aniline blue (BAB) staining in plant tissues. The section of the first stem internode of all the poplar samples had typical structure consisting of epidermis, cortex, annular vascular bundles, and pith (Fig. 5 and Additional file 1: Fig. S2). Vascular bundles contained primary phloem and lignified primary xylem with unlignified interfascicular cambium between the tissues. There was no significant difference in the development of lignified primary xylem among the samples (Fig. 5). However, lignified secondary xylem was enhanced in the middle internode section of *GbHDR1*ox poplars (Fig. 5). The lignified ring in the *GbHDR1*ox poplar is discontinuous, and this probably is the result of a local delay of cell wall lignification. Additionally, thick and dense secondary xylem was detected in the root base, which is a similar pattern observed in the middle internode. Furthermore, the BAB staining showed strong and extended signals in cross-section tissues in the *GbHDR1*ox transgenic poplars (Additional file 1: Fig. S2).

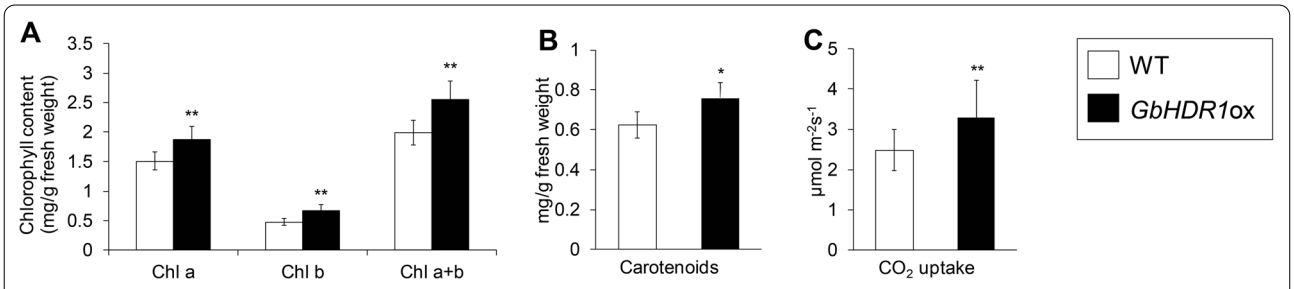

Fig. 4 Plant pigment contents and photosynthetic rate of WT and *GbHDR1*ox transgenic poplar plants. Contents of **A**: chlorophylls and **B**: carotenoids, and **C**: comparison of photosynthetic rate in WT (n = 3) and *GbHDR1*ox poplar plants (5 lines, n = 3 to 5). Error bar represents standard deviation. The asterisk symbol indicates **P < 0.01 and *P < 0.05

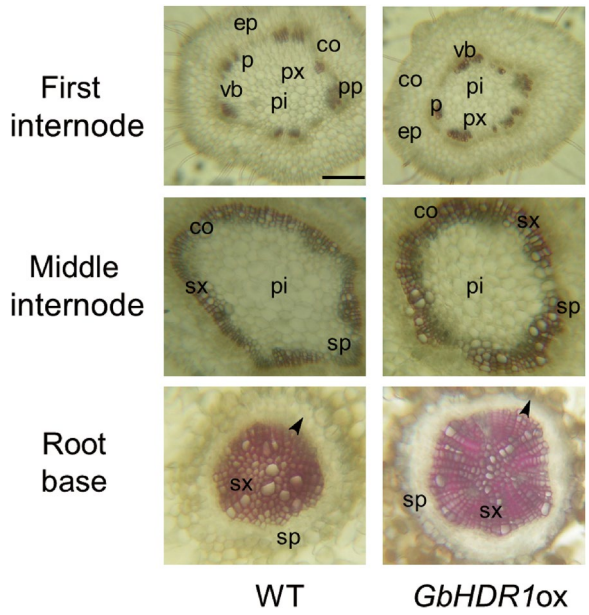

Fig. 5 Phloroglucinol staining of lignified tissue in WT of *GbHDR1*ox transgenic poplar plants. The black arrowheads in the figures denote casparian bands. co, cortex; ep, epidermis; p, parenchyma; pi, pith; pp, primary phloem; px, primary xylem; sp, secondary xylem; sx, secondary xylem; vb, vascular bundles. Scale bar = 100 μm

Discussion

Despite the extensive application values of terpenoids, there are limitations in the stable and large-scale supply of terpenoids to meet all the demands of industry. Recent advances in metabolic engineering with the combination of synthetic biology and systems biology have enabled researchers to bring various cell factories [2, 4, 24], and several successful cases have been utilized in the biotech industry [25].

The increase of precursor supply is one of the effective strategies to achieve the flux enhancement for producing desired chemical compounds [20, 24, 26]. Overexpression of *DXS*, the first committing step in MEP pathway, has been frequently conducted to enhance the metabolic precursor pools for terpenoid production. This strategy, indeed, increased carbon flux and improved terpene production [27–30]. However, the following steps of the MEP pathway often hamper plant growth [31]. In the case of *E. coli,* overexpression of *DXS* causes retarded growth and poor terpenoid production, which is attributed to the depletion of DXS substrates, glyceraldehyde 3-phosphate (G3P) and pyruvate [32–34]. Additionally, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MECP), a MEP pathway intermediate, is known to be a limiting step due to its efflux from the *E. coli* cell. Activation of downstream MEP pathway enzyme reduced this efflux to effectively channel metabolite flux to the pathway end products, IPP and DMAPP [35]. Moreover, MECP was reported as a retrograde-signaling metabolite, and it induced stress-related genes in Arabidopsis [36]. In plants, removal of G3P from the Calvin cycle due to *DXS*-overexpression was also suggested to contribute to the unsatisfactory growth of the *Arabidopsis* transformant [27]. It is possible, however, that overexpression of HDR would not draw G3P directly from Calvin cycle as opposed to overexpression of DXS, and thus positively contribute to the pool of isoprene units without critically disturbing carbon flux in the Calvin cycle. In addition, enhanced photosynthetic pigment contents due to the increased pool of isoprene units, in turn, would boost the photosynthetic rate to offset the partial siphoning of G3P from the Calvin cycle to the MEP pathway. For instance, the co-expression of tomato HDR and taxadiene synthase in Arabidopsis increases carotenoid and taxadiene levels [37]. It was observed that increased HDR activity caused improved plastidial terpenoid biosynthesis in *Arabidopsis* [37]. Therefore, we considered that overexpression of *HDR* that is downstream of MECP is thus a more rational approach to increase MEP pathway carbon flux by circumventing other negative cellular responses.

Generally in gymnosperm, HDR is known to present as isozymes, and our previous study also identified that *G. biloba* has a small family of three HDRs [15]. In fact, each member of the HDR family has been suggested to have a distinct role in terpenoid biosynthesis. Class 1 HDRs, including GbHDR1are suggested to be involved in primary household metabolism whereas class 2 HDRs relate to secondary metabolism. Class 2 HDRs are expressed in both a developmental and spatial specific manner while no such specificities occur in class 1 HDRs (Additional file 1: Fig. S3) [15, 18, 38, 39]. Recently, we investigated the impact of class 2 GbHDR by overexpressing the *GbHDR2* in tobacco plants and confirmed an increase of diterpenoid contents in *GbHDR2*ox tobacco plants [39]. Therefore, we expected that the GbHDR1 overexpression study would demonstrate not only the enhancement of carbon flux through the MEP pathway but also the specific functions of GbHDR1, that are related to primary metabolism.

The unavailability of Ginkgo transformation system to date justified the use of a heterologous plant system, which would lead to an evaluation of the application potential of *GbHDR1* in plant metabolic engineering. The poplar tree is an ideal plant model system with various advantages such as fast growth and high biomass. Wood composition of the plants is especially desirable in engineering plants for fuel uses. [40]. In addition, the triploid poplar trees used in this study are free from biosafety issues because they prevent gene escape due to infertility. Furthermore, extensive poplar studies

provide many accessible genetic tools and information to use the system straightforwardly for metabolic engineering research. Thus, we attempted to overexpress the *GbHDR1* in poplar to acquire stable transgenic plants of *GbHDR1*ox. In the initial growth stage, the *GbHDR1*ox poplar plants exhibited rapid growth accompanied by increased height and leaf number compared with the WT plants (Fig. 2). *GbHDR1*ox poplar plants formed winter buds late compared to the WT poplars, which suggests high tolerance against low temperatures in *GbHDR1*ox poplars. Poplars temporally cease shoot growth to avoid permanent growth termination by forming winter buds to protect from low and freezing temperatures.

The enhanced growth of *GbHDR1*ox plants suggested the increased carbon flux towards several terpenoid metabolites such as isoprene, GA, chlorophylls and carotenoids. For example, an increase of *IS* transcript levels suggested increased C5 isoprene precursor pools in *GbHDR1*ox poplars (Fig. 3B). Additionally, up-regulation of *IS* transcription indirectly reflected enhanced isoprene emission from leaves. The emission of isoprene is known to help plants overcome abiotic stress such as drought and temperature by protecting photosynthesis [41]. This fact suggests that the leaves of *GbHDR1*ox poplar can be an excellent platform to produce volatile terpenoids even under various stress conditions.

Enhanced GA (diterpene) biosynthesis in *GbHDR1ox* poplars could promote plant elongation, and increased levels of photosynthetic pigments such as chlorophylls (diterpene side chain) and carotenoids (tetraterpene) would stimulate photosynthesis. We thus analyzed the transcript levels of key genes in the biosynthetic pathway of these terpenoid metabolites as illustrated in Fig. 3A. The data in the present study clearly indicate that overexpression of *GbHDR1* in poplar brought about up-regulation of downstream genes in a feed-forward activation manner (Fig. 3A).

Wille et al. [42] suggested that the MEP pathway and the production of plastidial pigments have a significant degree of coordination at the gene expression level in a feed-forward manner. Thus, overexpression of DXS and HDR leads to increased levels of chlorophylls and carotenoids [27, 37]. The phytol side chain, a diterpene of MEP pathway origin, is an essential component of light-harvesting pigments, chlorophylls a and b, that anchors the pigments to the thylakoid membrane [43]. In the present study, overexpression of *GbHDR1* in poplar evoked upregulation of *CHS* and *CAO* and also resulted in enhanced accumulation of chlorophylls a and b. Plant carotenoids derived from the MEP pathway play a crucial role in photosynthesis as accessory pigments [44]. In *GbHDR1*ox transgenic poplar plants, we observed increased transcription levels of *GbPSY*, a key enzyme in carotenoid biosynthesis, with an concomitant increase of carotenoid contents.

It is evident in the present experiment that, besides simple law of mass action, up-regulation of downstream genes was operating in the *GbHDR1*ox plants to effectively drain the accumulated MEP pathway of end-products. During deetiolation of tomatoes, strong upregulation of HDR is accompanied by increased transcription of *PSY* [37]. Therefore, enhanced carotenoid accumulation due to *HDR* overexpression in the present work resembles the *PSY*-activating mechanism in chloroplasts. The upregulation of chlorophyll and carotenoid biosynthetic genes in turn increased chlorophyll and carotenoid contents in transgenic plants. Such increase of photosynthetic pigment contents would enhance the photosynthesis rate. Indeed, overexpression of *GbHDR1* led to elevated photosynthetic rates in the leaves of poplar (Fig. 4C).

Upregulation of *KS* and *GA20ox* transcription suggests elevated GA biosynthesis in the *GbHDR1*ox poplar plants (Fig. 3B). Because the biosynthesis of gibberellin is self-regulating, the effect of *GbHDR1* overexpression on gibberellin-catabolizing *GA2ox* must be considered, as well [45]. In this regard, it is very indicative that *GbHDR1* overexpression in poplar resulted in the upregulation of *KS* and *GA20ox* with concomitant *GA2ox* downregulation (Fig. 3B). The results from both plants thus strongly imply increased active GA content in the *GbHDR1*ox plants. An increase in bioactive GA level is known to promote plant growth [23] and the inhibition of growth cessation under short-day photoperiod in poplar trees [46]. Our data in *GbHDR1*ox poplars also displayed rapid growth and delayed terminal bud formation (Additional file 1: Fig. S1). Moreover, a previous GA20ox overexpression study in poplar proved that the increased bioactive GA level can improve the secondary growth in xylem fibers [23] and is consistent with our cross-section data of *GbHDR1*ox poplar that showed enhanced secondary xylem (Fig. 5 and Additional file 1: Fig. S2).

Increased photosynthesis and elevated GA levels are indicative of stem lengthening of stem in the *GbHDR*ox1 plants. Recently, it was shown that the presence of the N-terminal region of the GbHDR1 enzyme is responsible for the shift of the optimal pH of the enzyme toward the basic side so the enzyme operates under alkaline condition of chloroplast stroma [47]. Therefore, GbHDR1 best functions while the photosynthetic apparatus is operating. The close relationship of the enzyme with primary metabolism, embodied by expedited growth as shown in the present study, is suggestive.

Overexpression of *GbHDR1* would be an applicable strategy to boost accumulation of the target terpenes especially in various plant cell platforms. Plant system as a cell factory platform have many advantages for engineering the complicated plant terpene metabolic pathways [24, 26, 40]. Additionally, they use light for photosynthesis and no externally added sugars are required to synthesize secondary metabolites, which enable eco-friendly and sustainable way of plant origin chemical production. In particular, terpenoid production as fuels, which require vast amount of biomass, would be economically viable when using a plant system like the poplar cell factory.

Materials and methods

Plant materials and growth conditions

The non-flowering triploid poplar, *Populus glandulosa* BH1 (*Populus alba* × *Populus tremula* var. *glandulosa* BH1) was used in this study. Poplar seedlings were maintained in a culture room at 25 ± 2 °C under cool white fluorescent light for 16 h per day. The WT and transgenic poplars were moved from indoors to the outdoor nursery after 8 weeks of growing in pots, and they were grown in general outdoor conditions without any environmental protection.

Establishment of transgenic plants

A binary vector containing the kanamycin-resistance gene (pBI121, Clontech, USA) was used for the gene construction. The *GbHDR1*-coding region was amplified by PCR using primers designed to add *Xba*I and *Bam*HI sites to the 5′ and 3′ ends, respectively (Additional file 1: Table S1). The amplicon was inserted into the pBI121 binary vector under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Additional file 1: Fig. S4). The transformation vector was mobilized by freeze–thaw in *Agrobacterium tumefaciens* strains LBA4404 for poplar [48]. Poplar transformation was performed by co-cultivating the stem segments of the BH1 clone with LBA4404 strain carrying GbHDR1ox construct. The transformed calli were selected by kanamycin selection, and shoot and roots were regenerated in appropriate media with antibiotics before transferring to a 5-inch pot. The transgenic poplar plants were eventually transferred to the pots with sterilized potting mix and watered at 7-day intervals. The entire procedure for the establishment of transgenic poplars is briefly shown given with the information in Additional file 1: Fig. S5. To confirm the introduction of *GbHDR1* in the transformants, PCR reaction analysis was performed with cDNA of GbHDR1 transgenic poplars. A band of about 1450 bp, the expected size of *GbHDR1* ORF, was amplified from each *GbHDR1*ox transgenic poplar (Additional file 1: Fig. S4).

RNA extraction and quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from the leaves of the plants using the TRIzol (Life Technologies, USA) method [49]. The reverse transcriptase polymerase chain reaction (RT-PCR) was performed according to the protocol of Kim et al. [38]. To assess the transcript levels in poplar plants, *PaIS* (*Populus alba* x *Populus tremula* isoprene synthase), *PtKS* (*Populus trichocarpa* kaurene synthase), *PtGA20ox* (*P. trichocarpa* GA20 oxidase), *PtGA2ox* (*P. trichocarpa* GA2 oxidase), *PtCHS* (*P. trichocarpa* chlorophyll synthase), *PtCAO* (*P. trichocarpa* chlorophyll *a* oxygenase), and *PgPSY* (*P. trichocarpa phytoene synthase*) were used to design qRT-PCR primers (Additional file 1: Table S1). The qRT-PCR was carried out on a Rotor-Gene 2000 Real-Time Amplification System (Corbett Research, Australia) using the SYBR Green PCR system (Takara, Japan) according to the manufacturer's protocol. The thermal cycling profile consisted of: stage 1, 95 °C for 15 min; stage 2, 95 °C for 10 s and 52 °C for 30 s. Stage 2 was repeated for 40 cycles. The relative expression level was quantified using *P. trichocarpa* ubiquitin (*PtUBQ*) as a reference.

Analyses of chlorophylls, carotenoids, and photosynthetic rate

The leaves from 5-week old poplar plantlets were collected and ground with liquid nitrogen. The macerated paste was extracted with 40 mL of 80% acetone at 4 °C by shaking in the dark for 15 min in a 50-mL Falcon tube. Then the tube was centrifuged at 5000 rpm for 15 min at 4 °C before the optical density value of the supernatant was measured at 470, 647, and 663 nm. The contents of the pigments were calculated according to the equation given by Lichtenthaler and Buschmann [50].

Gas exchange measurements were made on fully opened leaves with the portable photosynthesis system Li-Cor 6400 (Li-cor, USA) 10 weeks after potting for poplar, grown in a growth chamber with light intensity at 77.7 μmol m^{-2} s^{-1} (Li-cor 250A Light Meter). Light for the measurement was provided by a Red/Blue LED light source (Li-Cor 6400-02B). Leaves were equilibrated at a photon irradiance of 100 μmol quanta m^{-2} s^{-1} for 30 min. During the experimental run, leaf chamber CO_2 concentration and humidity were maintained at 380 μmol mol^{-1} and 23 mmol mol^{-1}, respectively, and leaf temperature was maintained at 23 °C. The samples were taken from five plants belonging to the same line, and a *t*-test was performed for statistical validation.

Histochemical analysis of poplar plants

Poplar cuttings with one bud were aseptically grown on wood plant media (WPM) in test tubes for 65 days until the stem length reached about 10 cm. The stem was

sectioned under a dissecting microscope with a razor blade. For PG staining, sections were treated with one drop of concentrated HCl for 2–3 min followed by one drop of 5% phloroglucinol for 3–5 min. Lignified xylem walls appeared cherry red under a bright-field microscope (Leica DME with Nikon E5400 digital camera) [51]. BAB staining was carried out by adding one drop of 0.1% berberine hemisulfate to the section, which was allowed to stand for about 1 h followed by washing with water [52]. The staining was completed by immersion in one drop of 0.5% aniline blue for 30 min followed by washing with water. Lignified cell walls appeared as stagnant yellow, Casparian bands vivid yellow, and suberized cell walls brown on a fluorescent microscope (Olympus IX71 with excitation filter G 365 nm, absorption filter U-WB, dichromatic mirror DM500, compensation excitation filter BP450–480, and compensation absorption filter BA515). The photograph was taken with a digital camera RZ200C-21 (China).

Statistical analysis

The data obtained in this experiment were subject to statistical analysis. The student's t-test was performed by using SAS (version 9.1). Statistical significances were denoted as * ($P \leq 0.05$) and ** ($P \leq 0.01$).

Conclusion

In conclusion, we established *GbHDR1*ox transgenic poplar plants. The *GbHDR1* overexpression enabled improvement in terpenoid metabolic flux through the MEP pathway, which was supported by the up-regulation of transcript levels of downstream genes in terpene biosynthesis as well as increased terpenoid photosynthetic pigments. This study is noteworthy in terms of the functional study of gymnosperm HDR isozymes, and it also suggests an efficient way to boost terpenoid biosynthesis in plastids. Therefore, HDR is an ideal target for MEP pathway manipulation towards improved production of terpenoids and biomass in heterologous cell factories.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-022-00718-6.

Additional file1: Fig. S1 Winter bud formation and final height of poplar plants at growth cessation. After 2-month growing in outdoor nursery, winter bud was visible at the top apex in **A** WT poplar plants (n=3), but not in the **B** *GbHDR1*ox poplar plants (5 lines, n=3–5) and **C**: Final height after completion of winter buds both in WT and *GbHDR1ox* poplar plants. WT: Wild type, *GbHDR1*ox: *GbHDR1ox* transgenic poplars. The asterisk symbol indicates **P < 0.01 and *P < 0.05. **Fig. S2** Berberine-aniline blue staining (BAB) of WT and *GbHDR1ox* transgenic poplar plants. co, cortex; ep, epidermis; p, parenchyma; pi, pith; pp, primary phloem; px, primary xylem; sp, secondary xylem; sx, secondary xylem; vb, vascular bundles. Scale bar = 100 µm. *GbHDR1* **Fig. S3** Phylogenetic tree of plant HDRs. MEGA 6.0 software was used for sequence alignment and phylogenetic analysis with neighbor joining algorithm and Bootstrap with 1000 replications. (Le: *Lycopersicon esculentum*, Ls: *Lactuca sativa*, St: *Solanum tuberosum*, At: *Arabidopsis thaliana*, Vv: *Vitis vinifera*, Pot: *Populus trichocarpa*, Ap: *Adonis palaestina*, Hb: *Hevea brasiliensis*, Sr: *Stevia rebaudiana*, Hv: *Hordeum vulgare*, Os: *Oryza sativa*, So: *Saccharum officinarum*, Pt: *Pinus taeda*, Pd: *Pinus densiflora*, Gb: *Ginkgo biloba*, Ps: *Picea sitchensis*). **Fig. S4** Vector construction for *GbHDR1*ox transgenic plants (X: XbaI, B: BamHI). Direct PCR amplification of *GbHDR1* ORF sequence in the transgenic poplar plants, respectively. All lines carried *GbHDR1* gene. (M: maker, WT: wild type, GbHDR1ox: *GbHDR1*ox transgenic poplars). **Fig. S5** Scheme for the GbHDR1ox poplar regeneration procedures. CIM1 (callus induction medium 1): MS medium with 10 mM 2,4-D, 1.0 mM NAA, 0.1 mM BAP, sucrose (20 g/L), agar (15 g/L), and antibiotics (50 mg/L kanamycin and 100 mg/L cefotaxim) CIM2: CIM1 without antibiotics, SIM (stem induction medium): 10 mM Zeatin, 1.0 mM NAA, 0.1 mM BAP, sucrose and antibiotics, RIM (root induction medium):1/2 MS medium with agar and antibiotics. **Table S1**. Primers used in this study.

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Author contributions

M–KK, S-UK, and S-MK. designed the experiments. M–KK, J-YK, LH, CY, and ZJ. performed experiments. YIC. provided the poplar plants and supervised the management of transgenic poplar plants. YJP. helped with data analysis. M–KK, S-UK, and S-MK wrote the manuscript and all authors approved and commented on the manuscript. All authors read and approved the final manuscript

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

There is no competing interest.

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